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Mass Spectrometric Quantification of F_2-Isoprostanes as Indicators of Oxidant Stress

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1. Introduction

Free radicals derived primarily from molecular oxygen are believed to play a important role in a variety of disease processes including atherosclerosis, ancer, and neurodegenerative disorders *(1)*. Much of the evidence for this ssociation, however, is indirect largely because of limitations in techniques currently available to quantify free radicals or their products in biological systems *(2,3)*. This is a particular problem when assessing oxidant injury in vivo in humans.

Measures of lipid peroxidation are frequently employed to implicate free adicals in pathophysiological processes. Techniques include measurement of malondialdehyde, conjugated dienes, short chain alkanes, and lipid hydroperoxides, among others *(3)*. These methods suffer, however, from inherent problems related to sensitivity and specificity, particularly when applied to in vivo situations. In addition, artifactual generation of lipid-peroxidation products can occur ex vivo, and factors such as metabolism can markedly affect evels of various substances *(3)*.

Previously, we described a series of prostaglandin (PG)F_2-like compounds, ermed F_2-isoprostanes (F_2-IsoPs), that are produced in vivo in humans by a on-cyclooxygenase free radical-induced mechanism involving the peroxidaon of arachidonic acid *(4)*. Generation of these compounds initially involves ormation of four positional peroxyl radical isomers that endocyclize to orm PGG_2-like intermediates that are subsequently reduced to PGF_2-like compounds. Theoretically, four F_2-IsoP regioisomers are formed, each consist-

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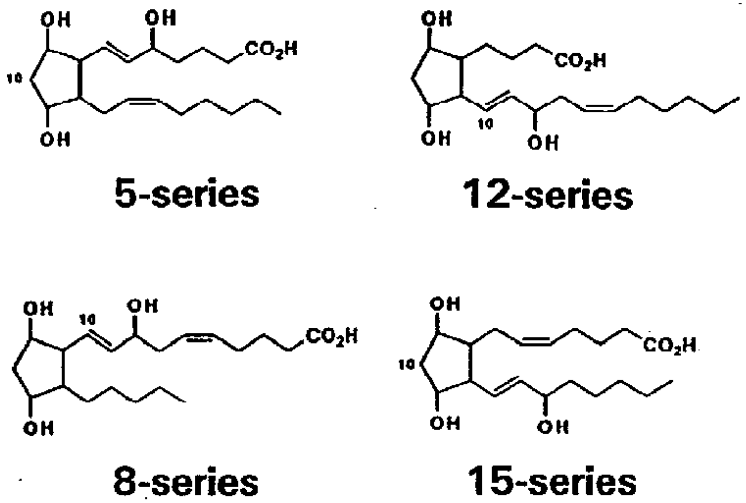

Fig. 1. Structure of the four F_2-IsoP regioisomers. Stereochemistry is not indicated.

ing of eight racemic diastereomers. Compounds are named as either 5-, 8-, 12-, or 15-series regioisomers according to which carbon the side chain hydroxyl group is attached **(Fig. 1)** *(5)*.

It has been found, over the past decade, that quantification of the F_2-IsoPs represents a reliable and useful approach to assess lipid peroxidation and oxidant stress in vivo *(6)*. For example, we have shown that the formation of F_2-IsoPs increases dramatically in animal models of oxidant stress and correlates with the degree of tissue damage. Further, F_2-IsoPs are present in easily detectable concentrations in normal human biological fluids, such as urine and plasma, allowing for a normal range to be defined and also for relatively small increases in these compounds to be measured accurately in settings of minimal oxidant stress. In addition, F_2-IsoPs can be detected in all types of biological fluids and tissues we have examined to date, providing an opportunity to assess the formation of these compounds at local sites of oxidant injury (*see* **Note 1**).

As noted, IsoPs are derived from arachidonic acid. The majority of arachidonic acid present in vivo exists esterified in phospholipids. We have previously reported that F_2-IsoPs are initially formed *in situ* from arachidonic acid esterified in phospholipids and are subsequently released preformed by the action of phospholipases *(7)*. This discovery raises an important issue regarding the assessment of IsoP formation in vivo in that depending on the studies that

one undertakes, total isoprostane production may be more accurately assessed by measuring levels of both free and esterified IsoPs. Further, the fact that F_2-IsoPs are formed *in situ* in phospholipids can be utilized in an advantageous way to assess oxidant injury in specific animal or human organs by analyzing levels of these compounds esterified in phospholipids from tissue biopsy specimens that may be obtained for diagnostic purposes.

The purpose of this chapter is to discuss methods for the analysis of F_2-IsoPs from biological sources by gas chromatography (GC)/negative ion chemical ionization (NICI) mass spectrometry (MS). Procedures are outlined for the analysis of both free and esterified F_2-IsoPs. F_2-IsoPs from biological sources can only be quantified as free compounds using GC/MS. Therefore to measure levels of IsoPs esterified in phospholipids, the phospholipids are first extracted from a tissue or fluid sample and subsequently subjected to alkaline hydrolysis to liberate the F_2-IsoPs from the phospholipids. Following hydrolysis, the free F_2-IsoPs are measured using the same techniques as free compounds in biological fluids. Thus, the following methods first outline the extraction and hydrolysis of F_2-IsoP-containing phospholipids from tissue or fluid samples. Subsequently, techniques for the analysis of free compounds are discussed.

2. Materials

2.1. Equipment

1. Blade homogenizer-PTA 10S generator (Brinkmann Instruments, Westbury, NY).
2. Table-top centrifuge.
3. Rotavap unit (organic solvent evaporator) (Brinkmann Instruments).
4. Tank of nitrogen gas.
5. Analytical evaporation unit (such as Meyer N-Evap, Organomation Associates Inc., Berlin, MA).
6. Microcentrifuge (Fisher Scientific, Pittsburgh, PA).
7. Water bath at 37°C.
8. Capillary gas chromatography column (DB-1701, Fisons Inc., Folsom, CA).
9. Gas chromatograph/mass spectrometer (with capabilities for negative ion chemical ionization mass spectrometry) (Hewlett Packard 5890 instrument, Palo Alto, CA).
10. Sep-Pak cartridges, C-18 and silica (Waters Associates, Milford, MA).
11. Disposable plastic syringes (10 mL).
12. Glass scintillation vials (20 mL).
13. Reactivials (5 mL) (Pierce Scientific, Rockford, IL).
14. TLC plates: LK6D silica (Whatman, Maidstone, UK).
15. Microcentrifuge tubes.
16. Conical glass centrifuge tubes (40 mL).
17. Conical-bottom flasks (100 mL).

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2.2. Reagents

1. Ultrapure water (triply distilled or its equivalent).
2. High-purity organic reagents: methanol, chloroform (containing ethanol as a preservative), ethyl acetate, acetonitrile, heptane, ethanol (Burdick and Jackson brand, VWR Scientific Inc., McGaw Park IL).
3. Butylated hydroxytoluene (BHT) (Aldrich Chemical Co., Milwaukee, WI).
4. Sodium chloride.
5. Triphenylphosphine (TPP) (Aldrich Chemical Co.).
6. Magnesium chloride.
7. Potassium hydroxide pellets.
8. HCl (American Chemical Society (ACS) certified or equivalent grade).
9. Pentafluorobenzylbromide (PFBB) (Aldrich Chemical Co.).
10. N,N′-Diisopropylethylamine (DIPE) (Aldrich Chemical Co.).
11. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco Inc, Bellefonte, PA).
12. Dimethyformamide (DMF) (Aldrich Chemical Co.).
13. Undecane (Aldrich Chemical Co.).
14. Phosphomolybdic acid (Sigma Chemical Co., St. Louis, MO).
15. Na_2SO_4 (anhydrous).
16. [$^2H_4$] 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) internal standard (Cayman Chemical, Ann Arbor, MI).

3. Method of Assay

3.1. Handling and Storage of Biological Fluids and Tissues for Quantification of F_2-Isoprostanes

As noted earlier, we have detected measurable levels of F_2-IsoPs in all biological fluids that we have examined. An important consideration when quantifying F2-IsoPs as a measure of lipid peroxidation, however, is the fact that these compounds can be artifactually generated ex vivo in biological fluids, such as plasma, in which arachidonyl-containing lipids are present (*6,8*). In this regard, we have previously reported that:

1. F_2-IsoPs can be generated ex vivo in biological fluids that are allowed to remain at room temperature for greater than 2 h or are stored at 0°C or –20°C.
2. Ex vivo generation does not occur, however, when fluids or tissues are stored at –70°C or colder for up to 1 yr.
3. Formation of IsoPs does not occur to a significant extent if biological fluids or tissues are processed immediately after procurement and if the free-radical scavenger BHT, and/or the reducing agent TPP, is added to the organic solvent during extraction of lipids.

Thus, samples obtained for analysis should either be processed immediately or stored at –70°C. In addition, fluids or tissues that are stored should be rapidly cooled to –70°C or lower; this can be accomplished by snap-freezing the samples in liquid nitrogen prior to storage at –70°C.

3.2. Extraction and Hydrolysis of F_2-IsoP-Containing Phospholipids

Esterified F_2-IsoPs can be analyzed in either biological fluids or tissues. The method for tissues differs somewhat from fluids and thus each is discussed separately (*see* **Note 2**).

3.2.1. Extraction of Tissue Lipids For Analysis of F_2-IsoPs

The method outlined herein is applicable to all animal and human tissue samples.

1. Weigh out 0.05–1 g of either fresh tissue or tissue frozen at –70°C.
2. Add tissue to 20 mL of ice-cold Folch solution (chloroform/methanol, 2:1 v/v) containing 0.005% BHT in a 40-mL glass centrifuge tube.
3. Homogenize tissue with blade homogenizer at full speed for 60 s.
4. Purge airspace in centrifuge tube with nitrogen and cap. Let solution stand at room temperature for 1 h to effect maximum extraction of lipids from ground tissue. Shake tube occasionally for several seconds during this period of time.
5. Add 4 mL aqueous NaCl (0.9%). Vortex or shake vigorously for 2 min.
6. Centrifuge on table-top instrument 800*g* for 10 min at room temperature.
7. After centrifugation, carefully pipette off top aqueous layer and discard. Remove the lower organic layer carefully from under the intermediate semisolid proteinaceous layer and transfer to a 100-mL conical bottom flask. Evaporate under vacuum on rotavap unit to dryness. When dry, immediately proceed to the hydrolysis step (**Subheading 3.3.**).

3.2.2. Extraction of F_2-IsoP-Containing Lipids From Biological Fluids

The technique used to extract F_2-IsoP-containing phospholipids from biological fluids differs somewhat from methods used for tissue because we have found that arachidonic acid in plasma is much more susceptible to autoxidation during lipid extraction than arachidonate in tissue samples (*see* **Note 2**). Thus, to suppress ex vivo oxidation of arachidonic acid in plasma, TPP, in addition to BHT, is added to the Folch solution.

1. Add 1 mL of a biological fluid to 20 mL ice-cold Folch solution containing 0.005% BHT and 5 mg TPP in a 40-mL glass conical centrifuge tube.
2. Shake vigorously for 2 min.
3. Add 10 mL 0.043% $MgCl_2$ and shake vigorously for 2 min.
4. Centrifuge 800*g* for 10 min at room temperature.

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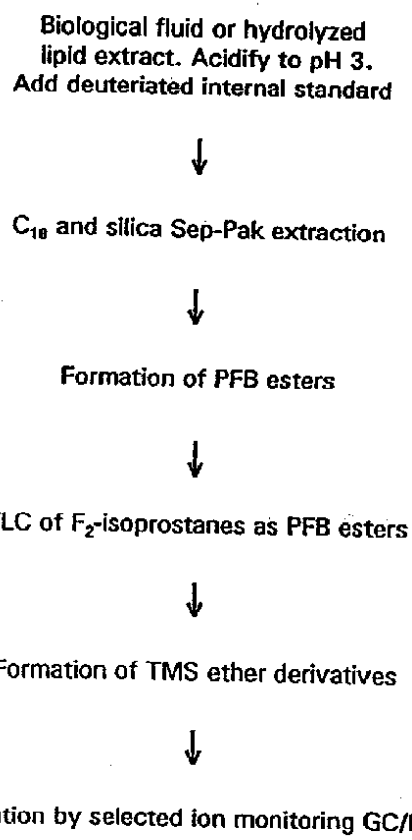

Fig. 2. Outline of the procedure for extraction, purification, derivatization, and MS quantification of F_2-IsoPs.

5. Separate lower organic layer from other layers and transfer to conical bottom flask (*see* **Subheading 3.2.1.**).
6. Dry organic layer at room temperature under nitrogen.
7. Proceed immediately to hydrolysis procedure (**Subheading 3.3.**).

3.3. Hydrolysis of Lipid Extracts

1. To lipid residue in conical flask, add 4 mL methanol containing 0.005% BHT and then add 4 mL aqueous KOH (15%). Purge flask with nitrogen and cap (*see* **Note 3**).
2. Incubate mixture at 37°C for 30 min.
3. After incubation, acidify the mixture to pH 3.0 with 1 *M* HCl. Then dilute the mixture to 80 mL with H_2O (pH 3.0) in preparation for extraction of free F_2-IsoPs. It is important to dilute the methanol in this solution to 5% or less to ensure proper column extraction of F_2-IsoPs in the subsequent purification procedure (**Subheading 3.4.**).

3.4. Purification and Derivatization of F_2-IsoPs

The assay method for purification and derivatization of free F_2-IsoPs in biological fluids or in hydrolyzed lipid extracts is outlined in **Fig. 2**. For purposes of discussion, the method used to purify and derivatize F_2-IsoPs in plasma is detailed below but the technique is equally adaptable to other biological fluids or tissue extracts.

1. Acidify 1–3 mL of plasma to pH 3.0 with 1 *M* HCl.
2. Add 1–5 pmol of deuteriated [$^2H_4$]15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) internal standard and vortex.
3. Apply mixture to a C-18 Sep-Pak column connected to a 10-mL syringe preconditioned with 5 mL methanol and then 5 mL pH 3.0 water.
4. Wash column with 10 mL pH 3.0 water and then 10 mL heptane.
5. Elute F_2-IsoPs from column with 10 mL ethyl acetate/heptane (50:50, v/v) into a glass scintillation vial.
6. Add 5 g anhydrous Na_2SO_4 to the vial and swirl gently. This step removes residual water from the eluate.
7. Apply eluate to silica Sep-Pak preconditioned with 5 mL ethyl acetate.
8. Wash cartridge with 5 mL ethyl acetate.
9. Elute F_2-IsoPs from silica Sep-Pak with 5 mL ethyl acetate/methanol (50:50, v/v) into reactivial.
10. Evaporate eluate under nitrogen.
11. To convert F_2-IsoPs to PFB esters, add 40 µL 10% (v/v) PFBB in acetonitrile and 10 µL 10% (v/v) DIPE in acetonitrile to residue for 30 min at room temperature.
12. Dry reagents under nitrogen and resuspend in 50 µL methanol.
13. Apply mixture to a lane on a silica TLC plate (LK6B) that has been prewashed with ethylacetate/ethanol (90:10, v/v). Chromatograph to 13 cm in a solvent system of chloroform/ethanol (93:7, v/v). For a TLC standard, apply approximately 2–5 µg of the methyl ester of $PGF_{2\alpha}$ to another TLC lane. Visualize the TLC standard by spraying the lane with a 10% solution of phosphomolybdic acid in ethanol and heating.
14. Scrape silica form the TLC plate in the region of the methyl ester of $PGF_{2\alpha}$ (R_f = 0.18) and adjacent areas 1 cm above and below.
15. Place silica in microfuge tube and add 1 mL ethyl acetate. Vortex vigorously for 30 s.
16. Pour off the ethyl acetate into another microcentrifuge tube taking care not to disrupt the silica pellet.
17. Dry organic layer under nitrogen.
18. Add 20 µL BSTFA and 6 µL DMF to residue.
19. Vortex well and incubate sample at 37°C for 20 min.
20. Dry reagents under nitrogen.

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21. Add 10 µL undecane (which has been stored over calcium hydride to prevent water accumulation). Sample is now ready for mass spectrometric analysis.

3.5. Quantification of F_2-IsoPs by GC/NICI MS

1. For the analysis of F_2-IsoPs, we routinely use a Hewlett Packard 5890 mass spectrometer interfaced with an IBM Pentium II computer system although other mass spectrometers can be utilized. The F_2-IsoPs are chromatographed on a 15 m DB1701 fused silica capillary column because we have found this GC columns gives excellent separation of individual regioisomers compared to other columns *(8)*. The column temperature is programmed from 190°C to 300°C at 20°C per min. Methane is used as the carrier gas for NICI at a flow rate of 1 mL/min.
2. The ion source temperature is 250°C, the electron energy is 70 eV, and the filament current is 0.25 mA. The ion monitored for endogenous F_2-IsoPs is the carboxylate anion m/z 569 (M-181, loss of $CH_2C_6F_5$). The corresponding carboxylate anion for the deuteriated internal standard is m/z 573.

3.6. Results: Application of the Assay to Biological Samples

1. **Fig. 3** shows the selected ion current chromatogram obtained from the analysis of F_2-IsoPs in the plasma of a rat 4 h after treatment with CCl_4 to induce an oxidant stress. The series of peaks in the upper m/z 569 chromatogram represent endogenous F_2-IsoPs. This pattern of peaks is virtually identical to that obtained from all other biological fluids and tissues examined thus far *(8)*. The peak in the lower m/z 573 chromatogram represent the $[^2H_4]15$-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) standard. Although all of the peaks noted in the upper m/z 569 chromatogram represent F_2-IsoPs, we routinely quantify the peak denoted by the asterisk (*). Using the ratio of the intensity of this peak to that of the internal standard, the concentration of F_2-IsoPs in the plasma sample in **Fig. 3** was calculated to be approx 2200 pmol/L, which is approx 20-fold above normal. Normal plasma levels of F_2-IsoPs in rats and humans are in the range of 60–170 pmol/L. Levels in other tissues and fluids have been reported elsewhere *(6,8)*. The F_2-IsoP assay in biological fluids is highly precise and accurate. The precision is ±6% and the accuracy is 96% (*see* **Notes 4, 5**).
2. In summary, this chapter outlines methods to assess lipid peroxidation associated with oxidant injury in vivo by quantifying concentrations of free and esterified F_2-Isops in biological fluids and tissues. Quantification of F_2-IsoPs appears to overcome many of the shortcomings associated with other methods to assess oxidant status, especially in vivo in humans. Thus measurement of F_2-IsoPs likely represents an important advance in our ability to assess the role of oxidant stress in human disease.

4. Notes

1. We have successfully used this assay to quantify F_2-IsoPs in a number of diverse biological samples (including urine, plasma, cerebrospinal fluid, and lung lavage fluid) and tissues (such as heart, muscle, brain, liver, and kidney).

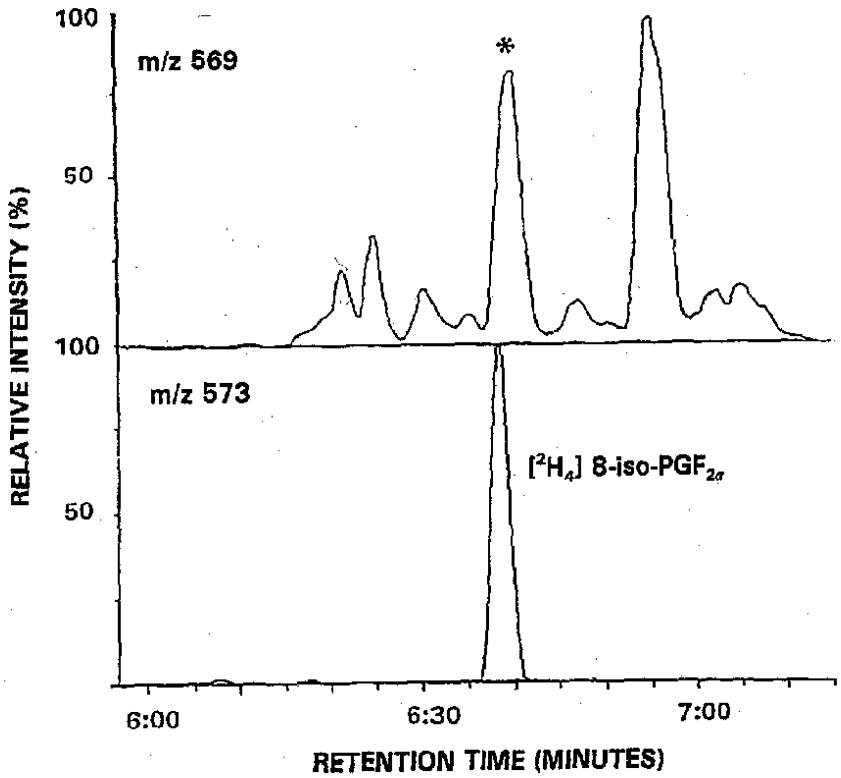

Fig. 3. Analysis of F_2-IsoPs in plasma from a rat 4 h after treatment with CCl_4 to induce lipid peroxidation. The peak in the m/z 573 chromatogram represents the $[^2H_4]15$-F_{2t}-IsoP internal standard. The peaks in the m/z 569 chromatogram represent endogenous F_2-IsoPs. The peak denoted by the asterisk is the one routinely used to quantify the F_2-IsoPs.

2. Regardless of the biological sample being processed to quantify esterified F_2-IsoPs, it is extremely important that the utmost care be taken during the extraction procedure to prevent ex vivo generation of F_2-IsoPs. This is more likely to occur if reagents contain significant trace metal contamination. To avoid this, reagents should be of very high purity. Further, the quality of water used during lipid extraction is extremely important and we routinely used triply distilled water or its equivalent. In addition, agents including BHT and TPP are added to suppress autoxidation of lipids. Finally, all glassware and plasticware are hand washed by us and rinsed with ultrapure water prior to use.
3. Hydrolysis of lipids in tissue or biological fluids should be performed immediately after lipid extraction to avoid potential autoxidation of arachidonate contained in the phospholipids.

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